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The M17 Leucine Aminopeptidase of the Malaria Parasite *Plasmodium falciparum*: Importance of Active Site Metal Ions in the Binding of Substrates and Inhibitors†

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ABSTRACT: The M17 leucine aminopeptidase of the intraerythrocytic stages of the malaria parasite *Plasmodium falciparum* (PfLAP) plays a role in releasing amino acids from host hemoglobin that are used for parasite protein synthesis, growth, and development. This enzyme represents a target at which new antimalarials could be designed since metalloaminopeptidase inhibitors prevent the growth of the parasites *in vitro* and *in vivo*. A study on the metal ion binding characteristics of recombinant *P. falciparum* M17 leucine aminopeptidase (rPfLAP) shows that the active site of this exopeptidase contains two metal-binding sites, a readily exchangeable site (site 1) and a tight binding site (site 2). The enzyme retains activity when the metal ion is removed from site 1, while removal of metal ions from both sites results in an inactive apoenzyme that cannot be reactivated by the addition of divalent metal cations. The metal ion at site 1 is readily exchangeable with several divalent metal ions and displays a preference in the order of preference $Zn^{2+} > Mn^{2+} > Co^{2+} > Mg^{2+}$. While it is likely that native PfLAP contains a Zn^{2+} in site 2, the metal ion located in site 1 may be dependent on the type and concentration of metal ions in the cytosolic compartment of the parasite. Importantly, the type of metal ion present at site 1 influences not only the catalytic efficiency of the enzyme for peptide substrates but also the mode of binding by bestatin, a metal-chelating inhibitor of M17 aminopeptidases with antimalarial activity.

Leucine aminopeptidases (LAPs) of the M17 peptidase family are metal-dependent exoproteases that hydrolyze N-terminal residues from proteins and peptides. Proposed functions for mammalian LAPs include protein degradation (*1*), antigen processing (*2*), and glutathione turnover (*3*). Abnormal LAP function has been associated with cataracts (*4*), cancer (*5*), and HIV infection (*6*).

LAPs are hexameric enzymes that contain two metal binding sites in the active site in each subunit (*7*−*9*). The relative affinities for binding a metal ion in each site of bovine lens leucine aminopeptidase (blLAP) are not equivalent; site 1 is termed the loosely bound or readily exchangeable site while site 2 is termed the tight-binding site (*10*). Both sites of the native bovine enzyme are occupied by a Zn^{2+} ion (*9*, *11*), but other divalent metal ions can also bind at these sites. Site 1 can be occupied by a number of metal ions such as Zn^{2+}, Co^{2+}, Mg^{2+}, and Mn^{2+} (*12*), but Co^{2+} is the only divalent metal cation other than Zn^{2+} that has been reported to bind at site 2 (*12*, *13*). Whatever the combination of metal ions in site 1 and site 2, both sites of blLAP must be occupied for proteolytic activity. In contrast, it has been reported that another dizinc LAP enzyme, porcine kidney leucine aminopeptidase (pkLAP), is active when the tight-binding site is occupied by Zn^{2+} but the loosely bound site is unoccupied. Despite this apparent difference in the two LAPs, kinetic studies have shown that changing the type of metal ion at the active site of both blLAP and pkLAP can influence enzyme activity (*8*, *12*, *14*), substrate specificity (*3*), and inhibitor binding (*15*).

Malaria is a disease caused by apicomplexan parasites of the genus *Plasmodia*, with *Plasmodium falciparum* being the most lethal of the four *Plasmodium* species infecting man. The annual death toll of this disease is 2−3 million, 85% of which occurs in sub-Saharan Africa, predominately in children under 5 years of age (*16*). The dramatic decline in the effectiveness of many

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antimalarial agents, such as chloroquine, means that there is a pressing need to discover and develop new antimalaria drug treatments. We have shown that the intraerythrocytic stages of malaria parasites express a cytosolic M17 leucine aminopeptidase (PfLAP) that is involved in the terminal stages of hemoglobin (Hb) digestion. Peptides generated by the degradation of Hb by endopeptidases within a specialized food or digestive vacuole are transported out to the cytoplasm for final breakdown to amino acids by PfLAP (*17*−*19*). The enzyme is a potential target for antimalarial drug development as dipeptide-like inhibitors that chelate metal ions within the active site of PfLAP prevent *P. falciparum* growth both *in vitro* and *in vivo* (*20*, *21*). We have produced a recombinant enzyme (rPfLAP) (*22*) which forms a hexameric structure like the mammalian enzymes, and each monomer contains the conserved residues Lys250, Asp255, Asp273, Asp332, and Glu334 that are known to be involved in metal ion binding in blLAP (*9*). Therefore, in the present study we investigated the importance of the active site-bound metal ions of the malaria enzyme in substrate and drug binding.

MATERIALS AND METHODS

Materials. Bestatin, EDTA,[1] and Leu-NHMec were purchased from Sigma Chemical Co. (Sydney, Australia). Ni-NTA was purchased from Qiagen (Victoria, Australia).

Purification of Recombinant PfLAP. rPfLAP was expressed in insect cells and purified by affinity chromatography on Ni-NTA agarose as previously described (*22*). The enzyme was subsequently dialyzed into 50 mM Tris-HCl buffer, pH 8.0, containing 10 μM $ZnCl_2$. Purity of the enzyme was checked on a 12% SDS−PAGE gel.

Inhibition of rPfLAP by EDTA. Inhibition of rPfLAP by EDTA was carried out in 96-well plates using a KC-4 Bio-Tek plate reader. Enzyme (30 nM) was incubated with metal chelator (5 nM−20 mM) in 50 mM Tris-HCl buffer, pH 8.0, for 5 min before addition of Leu-NHMec (20 μM). Rates of hydrolysis were recorded by monitoring the increase in fluorescence over 30 min at 37 °C. Inhibition was observed by plotting percent relative activity against log of the chelator concentration, where 100% relative activity is the rate in the absence of inhibitor.

Binding of Divalent Metal Ions to Site 1. Divalent metal ions (5 nM−0.1 M) in the form of their metal chlorides were incubated with 30 nM enzyme for 5 min before addition of substrate (10 μM). Since binding of divalent metal ions to site 1 occurred over a wide concentration range of ligand, the data were best visualized on semilog plots (*23*). The dissociation constant K_d was calculated by fitting the data to the equation

$$-\log [L] = -\log K_d + \log\left(\frac{1-v}{v}\right)$$

where L is the free ligand and v is the percent relative activity and is a measure of the fraction of bound sites divided by total sites.

Enzyme Efficiency of Metal Hybrids. Zn^{2+}, Mn^{2+}, Co^{2+}, and Mg^{2+} were added to PfLAP(Zn_) at concentrations to fully occupy site 1 (10 μM, 5 mM, 5 mM, and 25 mM, respectively). Rates of Leu-NHMec hydrolysis were then recorded over a range of substrate concentrations (5−625 μM). k_{cat} and K_m values were calculated from the Michaelis−Menten plots.

[1]Abbreviations: EDTA, ethylenediaminetetraacetic acid; Leu-NHMec, leucine-4-methylcourmarinyl-7-amide; $ZnCl_2$, zinc chloride.

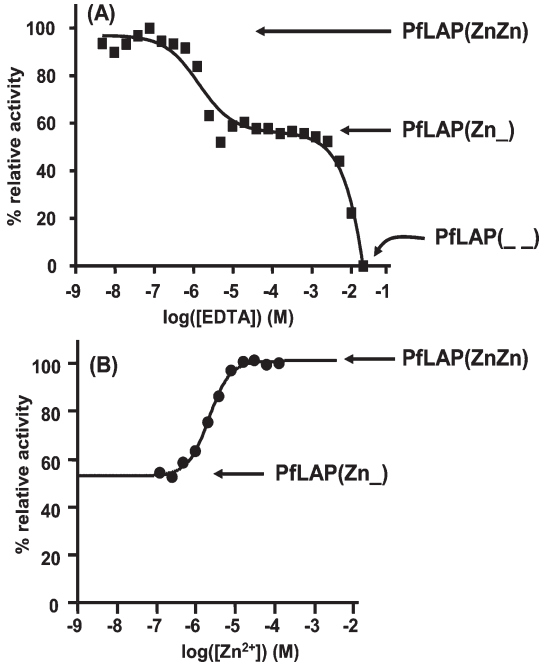


FIGURE 1: (A) Inhibition of rPfLAP(ZnZn) by EDTA. Enzyme (30 nM) was incubated with EDTA (5 nM−20 mM) in pH 8.0 buffer containing 10 μM free Zn^{2+} for 5 min before addition of substrate (20 μM). (B) Binding of Zn^{2+} to site 1 of PfLAP(Zn_). Zn^{2+} (100 nM−0.5 mM) was incubated with 30 nM enzyme with an unoccupied site 1 PfLAP(Zn_) before addition of substrate H-Leu-NHMec (20 μM).

Inhibition of rPfLAP by Bestatin. Inhibition of rPfLAP (ZnCo) by bestatin was carried out as follows. rPfLAP(Zn_) was incubated in 50 mM Tris-HCl, pH 8.0, containing 2 mM Co^{2+} for 2 min before adding the enzyme solution to wells containing substrate and inhibitor (2−100 nM). The enzyme concentration was 5 nM, and the substrate concentration was varied (5−100 μM). Rates of fluorescence release were followed for 50 min until a linear steady-state rate v_s was observed, and the K_i* value was calculated from a Dixon plot of $1/v_s$ versus [I] (*24*). Progress curves were fitted to an integrated rate equation describing slow-binding inhibition (*25*).

Inhibition of enzyme with an unoccupied site 1 was carried out in an identical manner except Co^{2+} was omitted from the assay. This time the enzyme concentration was 20 nM, the inhibitor concentration range was 20−500 nM, and the substrate concentration was varied from 50 to 625 μM. Progress curves in the presence of bestatin were linear.

RESULTS AND DISCUSSION

Identification and Characterization of Site 1 and Site 2 Metal Ions in PfLAP. Titrations against rPfLAP with the metal chelator EDTA revealed that the enzyme possesses two metal ion binding sites that are analogous to the readily exchangeable site (site 1) and the tightly bound site (site 2) found in blLAP and pkLAP. A PfLAP solution (30 nM) containing 10 μM Zn^{2+} is fully occupied at both binding sites and was designated as having 100% activity and termed PfLAP (ZnZn) (*26*). When EDTA was added to this solution over a wide concentration range (5 nM−20 mM), we found that, at equimolar concentrations of EDTA and free zinc, enzyme activity decreased to 57% (Figure 1A). We interpreted this as removal of Zn^{2+} from site 1, PfLAP(Zn_), and it shows that the malaria enzyme can function when this site is unoccupied.

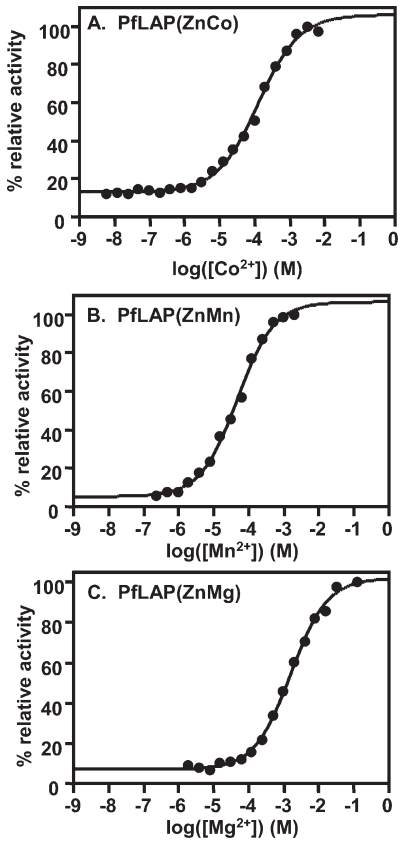


FIGURE 2: Binding of (A) Co^{2+}, (B) Mn^{2+}, and (C) Mg^{2+} to site 1 of PfLAP(Zn_). Co^{2+}, Mg^{2+}, and Mn^{2+} (100 nM−0.5 mM) were incubated with 30 nM enzyme with an unoccupied site 1 before addition of substrate H-Leu-NHMec (20 μM).

Addition of Zn^{2+} ion to PfLAP(Zn_) to reoccupy site 1 resulted in a return of the enzyme's activity to 100% (Figure 1B). This is similar to the observations of Lin et al. (*14*) with pkLAP, which can cleave substrates when a metal ion is bound only in site 2, but differs to those of Allen et al. (*12*), who found that blLAP requires a metal ion bound in both site 1 and site 2 for activity.

When the concentration of EDTA was raised to 20 mM, the metal ion occupying the tight binding site 2 of PfLAP was removed to form PfLAP(_ _) and resulted in complete loss of activity (Figure 1A). An inactive apoenzyme prepared by dialyzing PfLAP against 20 mM EDTA could not be reactivated, suggesting that the site 2 metal ion is essential to maintain the atomic structure of a functional active site. This is consistent with observation with both blLAP and pkLAP (*7*, *12*).

Binding of Zn^{2+}, Co^{2+}, Mg^{2+}, and Mn^{2+} to Site 1. We prepared an enzyme with the site 1 unoccupied, PfLAP(Zn_), and then assessed the ability of various divalent ions including Zn^{2+}, Co^{2+}, Mg^{2+}, and Mn^{2+} to bind this site and reactivate the enzyme to 100% (activity was monitored using the fluorogenic peptide Leu-NHMec as substrate). The metal content of the monozinc form of the enzyme PfLAP(Zn_) was measured by inductively coupled plasma mass spectrometry (ICP-MS) and shown to contain 0.8 mol of zinc/mol of enzyme (data not shown). The percent relative activity shown in Figure 2 is a measure of the fraction of bound sites, whereby 100% relative activity corresponds to enzyme with all site 1 binding sites fully occupied. We assume that at 100% activity the concentration of free metal ligand approximates the concentration of ligand added since it is >100-fold in excess over the enzyme concentration in all experiments. (The lowest dissociation constant (K_d) is 2.3 μM for Zn^{2+}, see below, and the enzyme concentration is 30 nM.)

Table 1: Dissociation Constants K_d for Binding of Zn^{2+}, Co^{2+}, Mn^{2+}, and Mg^{2+} to rPfLAP(Zn_)

metal ion	K_d (μM)
Zn^{2+}	2.3 ± 0.1
Mn^{2+}	47 ± 3
Co^{2+}	130 ± 5
Mg^{2+}	1600 ± 84

In the case of all metal ions tested PfLAP activity increased over a wide concentration range of divalent metal ions to reach maximum activity. Dissociation constants were calculated as the midpoint on semilog plots and allowed us to determine that site 1 bound metal ions with the preference $Zn^{2+} > Mn^{2+} > Co^{2+} > Mg^{2+}$ (Table 1). Zn^{2+} bound tightest to site 1 (K_d = 2.3 μM), and activity of PfLAP(Zn_) increased ~2-fold as site 1 became fully occupied with Zn^{2+} (see Figure 1B). In contrast, Mn^{2+}, Co^{2+}, and Mg^{2+} required higher concentrations to fill site 1, K_d = 47, 130, and 1600 μM, respectively (Table 1). However, the increase in PfLAP(Zn_) activity was 10−20-fold when occupied by these metal ions (Figure 2). The increase in activity on addition of metal ion solutions was not due to changing the ionic strength of the assay solution since KCl at similar concentrations (10 μM−0.5 mM) did not alter enzyme activity. Also, enzyme activity decreased when divalent metal ions were added at >20 mM concentration in the form of their metal chlorides, which was most likely due to the increase in ionic strength (results not shown).

To confirm that the metal ion in site 1 does not remain bound when excess metal ions are removed from the enzyme solution, PfLAP(Zn_) was preincubated in 1 mM Co^{2+} and then diluted into the assay solution so that $[Co^{2+}] \ll K_d$. The activity of the enzyme was identical to the activity of enzyme that had not been preincubated with Co^{2+}, i.e., exhibited no increase in activity (not shown). However, if Co^{2+} was added to the diluted PfLAP (Zn_), the enzyme increased activity similar to the titration curve shown in Figure 2A. These data demonstrate that the bound metal ion in site 1 of PfLAP dissociates instantaneously upon dilution but is also readily replaced.

The Metal Ion Occupying Site 1 Can Influence Enzyme Substrate Kinetics. To determine the effect of changing the metal ion in site 1 on enzyme efficiency, the catalytic parameters k_{cat}, K_m, and k_{cat}/K_m were calculated for each of the four metal hybrids PfLAP(ZnZn), PfLAP(ZnMn), PfLAP(ZnCo), and PfLAP(ZnMg) against the substrate H-Leu-NHMec. The Michaelis−Menten plots reveal obvious differences in enzyme efficiency between these hybrids (Figure 3), and the kinetic data presented in Table 2 show that the PfLAP(ZnZn) hybrid had the lowest efficiency for the substrate Leu-NHMec (k_{cat}/K_m = 7.5 M^{-1} s^{-1}). Most noticeable is the dramatic 11-fold increase in k_{cat}/K_m for the PfLAP(ZnCo) hybrid compared to the PfLAP (ZnZn) enzyme, mainly due to a much lower K_m value. The k_{cat}/K_m increased ~4-fold when Mn^{2+} and Mg^{2+} were bound in site 1. Increased enzyme efficiency toward chromogenic substrates has previously been observed when Co^{2+}, Mg^{2+}, and Mn^{2+} replaced Zn^{2+} in site 1 of blLAP or pkLAP (*27*).

The Site 1 Metal Ion Influences the Mode of Inhibition by Bestatin. Bestatin is an antibiotic and natural analogue of the dipeptide Phe-Leu derived from the fungus *Streptomyces olivoretticuli*. It is a well-known inhibitor of mammalian

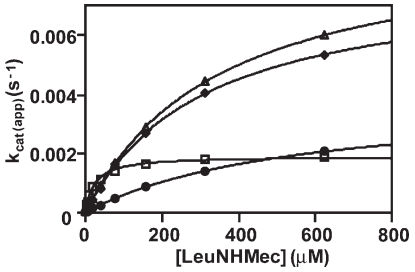
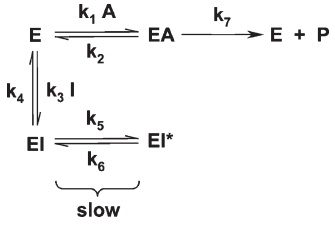


FIGURE 3: Michaelis−Menten plots for hydrolysis of H-Leu-NHMec by PfLAP(ZnZn) (●), PfLAP(ZnCo) (□), PfLAP(ZnMn) (◆), and PfLAP(ZnMg) (△). To fully occupy site 1, PfLAP(Zn_) (30 nM) was incubated with Zn^{2+}, Mn^{2+}, Co^{2+}, and Mg^{2+} (10 μM, 5 mM, 5 mM, and 25 mM, respectively). The enzyme solutions were then added to wells containing a wide concentration range of substrate spanning K_m (5−625 μM).

Table 2: Catalytic Parameters k_{cat}, K_m, and k_{cat}/K_m for Hydrolysis of H-Leu-NHMec by rPfLAP(Zn_), rPfLAP(ZnZn), rPfLAP(ZnCo), rPfLAP (ZnMn), and rPfLAP(ZnMg)

rPfLAP hybrid	$k_{cat} \times 10^3$ (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
Zn_	2.2 ± 0.2	770 ± 40	2.9
ZnZn	3.8 ± 0.2	510 ± 20	7.5
ZnCo	1.9 ± 0.1	23 ± 1	82
ZnMn	8.0 ± 0.3	310 ± 10	26
ZnMg	9.4 ± 0.4	350 ± 20	27

Scheme 1: Mechanism for Slow-Binding Inhibition of Bestatin to rPfLAP



LAPs (*28*−*31*). Bestatin also inhibits PfLAP and prevents *P. falciparum* malaria growth in culture (*20*, *32*, *33*). The enzyme and inhibitor readily form an initial EI complex which then undergoes a slow step to form a more tightly bound EI* complex (Scheme 1). One explanation for the slow-binding step proposed by Kim and Lipscomb (*10*) is that on entering the enzyme active site bestatin first coordinates with the site 1 metal ion before settling into a tighter complex that coordinates the site 2 metal ion.

The inhibition of PfLAP by bestatin in the presence of 1 mM Co^{2+} (i.e., Co^{2+} in site 1) is time-dependent, and the rate of substrate hydrolysis in the presence of inhibitor decreased over time to a steady-state rate indicating slow-binding inhibition (Figure 4A). By contrast, omitting Co^{2+} from the PfLAP−bestatin inhibition assay excluded the slow step resulting in linear rates of substrate hydrolysis in the presence and absence of inhibitor (Figure 4B). Our results, therefore, agree with the proposal of Kim and Lipscomb (*34*) regarding the importance of metal ions in the slow-binding characteristic of bestatin. Inhibition of rPfLAP(ZnCo) by bestatin (K_i^* = 23.0 ± 3.7 nM) was 5-fold greater than inhibition of rPfLAP(Zn_) (K_i = 107 ± 12 nM), showing that bestatin can form a tighter complex with PfLAP when both site 1 and site 2 are occupied.

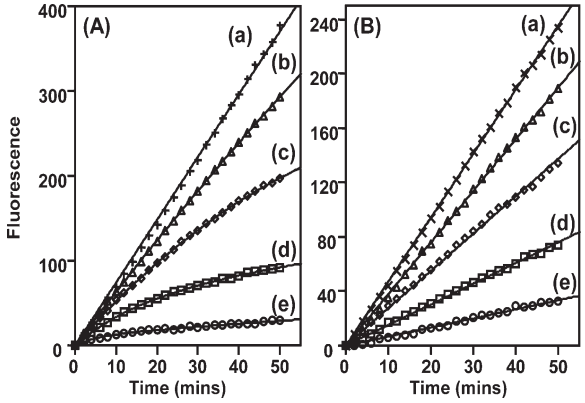


FIGURE 4: Inhibition of PfLAP by bestatin. (A) Progress curves for hydrolysis of 10 μM H-Leu-NHMec by 5 nM PfLAP(ZnCo) at pH 8.0 in the presence of bestatin: (a) 10 nM, (b) 20 nM, (c) 80 nM, and (d) 200 nM. (B) Progress curves for hydrolysis of 10 μM Leu-NHMec by 20 nM PfLAP(Zn_) at pH 8.0 in the presence of bestatin: (a) 20 nM, (b) 80 nM, (c) 200 nM, and (d) 500 nM.

Slow-binding inhibition was also observed for LAP(ZnZn), LAP (ZnMn), and LAP(ZnMg) (not shown).

The metal ion occupying both binding sites in the native malaria PfLAP is most likely zinc, as found for both blLAP and pkLAP. The dissociation constant for Zn^{2+} at site 1 (2.3 μM) is much lower than for Mn^{2+}, Co^{2+}, and Mg^{2+} (Table 1), and therefore, this metal ion would be more readily picked up from the surrounding cytoplasmic milieu. However, k_{cat}/K_m values show that of all four metal hybrids studied PfLAP(ZnZn) had the lowest catalytic efficiency at low substrate concentrations (Table 2). By contrast, under saturating conditions PfLAP(ZnZn) is most efficient; PfLAP(ZnZn) is also more active than PfLAP (ZnCo) at [H-Leu-NHMec] > 600 μM (see k_{cat} values in Table 2). Accordingly, intracellular zinc concentration may be an important regulator of native PfLAP activity particularly since saturated PfLAP(ZnZn) exhibits linear kinetics against peptide substrates in the range 1−600 μM (see Figure 3). Considering that the concentration of tetrameric Hb in red blood cells of healthy individuals is ~50 μM, it is probable that the malaria cytosolic concentration of Hb-derived peptide substrates fluctuates within a range where activity of the binuclear zinc enzyme is highest.

Nevertheless, PfLAP can accommodate divalent metal ions other than Zn^{2+} in site 1, and since enzyme efficiency is influenced by the occupying metal, especially at low substrate concentrations, this may offer another level of regulation. In this respect, magnesium may play an important role as Atamna and Ginsburg (*35*) estimate that the concentration of Mg^{2+} in parasites is particularly high, in the order of 4.6 mM (which agrees with measurements of 5.6 mM obtained by ICP-MS in our laboratory, not shown). Furthermore, elevated levels of serum magnesium have been observed in patients with acute *P. falciparum* malaria infection (*36*).

PfLAP plays a role in regulating the pool of amino acids in the parasite cytosol (*22*). These are essential to parasite protein anabolism and, hence, growth and development. PfLAP may also maintain levels of intracellular leucine, which is abundant in Hb. Leucine is required as it can be exchanged for serum isoleucine, an amino acid absent in human Hb (*37*). Indeed, treatment of parasite cultures with the metal chelator dipicolinic acid inhibits the incorporation of [^{3}H]isoleucine into parasite proteins (*38*). Our recent data show that inhibitors of PfLAP that

chelate the two metal ions within the active site have potent antimalarial activity (*21*). Our present report highlights the importance of understanding the critical role active site metal ions play in substrate and inhibitor binding before we embark on the search and development of a new class of antimalarial compounds targeting metalloaminopeptidases.

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